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13. ABSTRACT (Maximum 200 words) The purpose of this study is to evaluate the role of the growth factor Cripto-1 (CR-1) in mammary development and carcinogenesis. During the second year of this grant, we discovered that alteration of CR-1 levels affects colony formation of +SA cells. Two additional CR-1-specific ribozymes were designed and tested in vitro. Due to problems with the retroviral vectors, we are creating adenoviral vectors for the new ribozymes and CR-1. These vectors may not be compatible with the tasks in T.O. 1, due to a lack of integration into the developing mammary epithelium. In order to perform these studies, we are attempting to purify native CR-1 for direct delivery to the developing gland. We have had some success in purifying native CR-1, but the quantities are still low. We were able to obtain enough to show that there are at least two CR-1 binding proteins present in murine and human mammary epithelial cells. The next year will be used to finish the development of the adenoviral delivery systems for the ribozymes and CR-1 relevant to T.O. 2, 3, and 4. We will also be expending some effort on purification schemes to accomplish the tasks in T.O. 1.				
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FOREWORD

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Introduction

The format for this report is as follows: selected sections of the introduction have been included for reference. Progress to date has been reported in indented paragraphs under the appropriate section of the proposal. Where appropriate, achievement of specific tasks in the statement of work has been addressed.

The incidence rate for breast cancer generally increases with advancing age [1]. It remains unclear which variables may be involved in this relationship, though one candidate is a newly discovered member of the epidermal growth factor family, Cripto-1 (CR-1) [2-4]. Previous results from our laboratory indicate that CR-1 expression is increased in old mammary tissue [5], and that treatment of old mammary cells with tamoxifen *in vitro* reduces CR-1 mRNA levels (our unpublished results). **These results suggest that CR-1 may be involved in carcinogenesis in older mammary tissue and also in the hormonal regulation of breast cancer.**

CR-1 is expressed at low levels in adult murine, spleen, heart, lung, brain, and breast [6, 7], and at elevated levels during fetal development [6] and during the growth of mammary epithelium during adolescence and pregnancy [7]. CR-1 is expressed in human breast cancer tissues [8, 9] and cell lines (both estrogen receptor positive and negative, [8]), as well as in colorectal tumors [10] and pancreatic cancer cells [11]. In two separate studies, 75-82% of breast cancer tissue samples were positive for CR-1, whereas 0-13% of adjacent, non-involved tissue samples were positive [8, 9], implying that CR-1 may be involved in growth regulation of breast cancer cells. In addition, overexpression of CR-1 in some immortalized murine mammary epithelial cells is sufficient to cause an increase in anchorage-independent growth, but not sufficient for tumor formation in nude mice [12]. Finally, treatment of CR-1-expressing teratoma cells with retinoic acid results in differentiation of the cells and shutoff of CR-1 transcription [2]. **All of the currently published data are correlative and provide only circumstantial evidence for a functional role of CR-1 in breast cancer.**

CR-1 plays a significant role in the neoplastic phenotype of some human colon cancer cell lines [13]. Treatment of CR-1-positive colon cancer cells with antisense RNA (either directly *in vitro* or by infection with a retroviral antisense expression vector) resulted in decreased CR-1 protein levels, reduction in both anchorage-dependent and -independent growth, and reduced ability to form tumors in nude mice [13]. **These results suggest that reduction, or better yet, elimination of CR-1 expression in CR-1-positive tumor cells may have a significant therapeutic effect.**

Hypotheses

1. Proper expression of Cripto-1 (CR-1) is required for the normal development of the mammary gland epithelial structure. (T.O. 1)
2. Overexpression of CR-1 will increase tumorigenicity of non-tumorigenic or moderately tumorigenic mammary epithelial cell lines. (T.O. 2)
3. Underexpression of CR-1 will decrease tumorigenicity of highly or moderately tumorigenic cell lines. (T.O. 2)
4. Efficient delivery of a ribozyme or other therapeutic gene to a developing tumor may be accomplished by injecting a retroviral vector at the tumor location. (T.O. 3)
5. Delivery of a retroviral vector containing a CR-1-specific ribozyme to an established CR-1-expressing tumor *in vivo* will result in the regression of that tumor. (T.O. 4)

Technical Objectives

1. Determine the role, if any, for CR-1 expression in the developing mouse mammary gland.
 - a. Infect progenitor cells of the mammary epithelial tree with CR-1 over- or under-expressing retroviral vectors; examine mammary epithelial morphology.
 - b. Quantify changes in CR-1 protein expression in the mammary gland.
2. Analyze the role of CR-1 in the neoplastic growth of murine mammary epithelial cell lines (CL-S1, -SA, and +SA) *in vitro* and *in vivo*.
 - a. Look for changes in *in vitro* growth rate and anchorage-independent growth in cells over- and underexpressing CR-1.
 - b. Examine tumor and metastasis production in syngeneic hosts *in vivo* in cells over- and underexpressing CR-1.
3. Establish a model system for the delivery of a gene to a mammary tumor using a retroviral vector.
 - a. Use the defective (non-replicative) retroviral vector, CA1, which expresses β -galactosidase (β -gal), to infect tumor cells transplanted into epithelium-free mammary fat pads. Determine both the optimum conditions for infection of tumor cells and the largest tumor size which can be effectively infected.
4. Use a retroviral vector for treatment of mammary tumors *in vivo*.
 - a. Use the retroviral ribozyme construct from T.O. 1 to treat mammary epithelial cell tumors. Generate mammary tumors and then use the optimum conditions determined in T.O. 3 for delivery of the ribozyme. Score alterations in tumor growth and the percentage of tumor and non-tumor cells infected.

Body

Results to date are indented from the previously submitted procedures and methods.

T.O. 1a. The CA1 retroviral vector (gift from P.A.W. Edwards, University of Cambridge) will be used for these experiments. The packaged vector will be produced in GP+E-86 cells, a mouse-specific retroviral packaging cell line [14]. Viral supernatants will be harvested, then filtered through 0.45 μ m membranes to remove cells and cellular debris. If necessary, the virus may be concentrated by centrifugation through a 20% sucrose cushion at 34,000 $\times g$ for 6 hr, followed by resuspension in 1/100 of the original volume [15]. Viral titers will be determined by infection of +SA cells with serially diluted virus stocks, followed by staining with x-gal (or selection with G418). The number of blue cells (or G418^R colonies) will indicate the number of infectious virions per unit volume in the concentrated supernatant.

The epithelial ductal structure in the murine mammary gland does not develop until 3-6 weeks after birth. Therefore it is possible to produce epithelium-free mammary glands by removing the epithelial rudiment at 2-3 weeks of age [16]. It has been demonstrated that mammary epithelial cells transplanted into such a "cleared" gland will form a ductal structure, and that the mammary cells may be modified *in vitro* prior to implantation [16, 17]. Our results indicate that it is also possible to modify the epithelial rudiment itself by infection with a retrovirus.

Thirty mice (60 mammary glands) will be infected with either 2500 cfu (25 μ l at 10^5 cfu/ml) of CA1 (β -gal only), CA1CR (expressing CR-1), or CA1CRZ (expressing a CR-1-specific ribozyme) in serum-free Dulbecco's modified Eagle's medium (DME) with 80 μ g/ml polybrene; or treated with 25 μ l DME plus 80 μ g/ml polybrene alone. The infection will be performed by anaesthetizing the mouse, reflecting the skin to expose the #4 (inguinal) mammary gland, and injecting a 25 μ l virus suspension into the gland. The wound will be closed with surgical clips, and aseptic technique will be maintained throughout as recommended by the WSU IACUC Guidelines for Survival Rodent Surgery. The use of both #4 glands will allow us to directly compare two treatments in the same mouse, any quantitative data may then be compared using a paired T-test; otherwise quantitative comparisons will be made among all groups using ANOVA and Fisher's PLSD. The initial chosen pairings will be CA1 with CA1CR and CA1CRZ with DME. If other specific pairings are warranted by the findings, the experiment can be repeated.

Three mice from each pairing (thus 3 glands from each treatment) will be sacrificed after 1 week, and every week thereafter for a total of 5 weeks. This schedule will allow us to follow the development of the mammary epithelium and evaluate the effect of the treatments throughout the process of gland development. In the future, it may be informative to examine the morphology of transduced glands in pregnant or aged mice, due to the higher observed levels of CR-1 expression in those glands. Changes in gland morphology may be qualitatively assessed by observation of the whole mounts. If desired, glands can be dissolved out of Permunt with xylene and then embedded in paraffin for sectioning. Changes in CR-1 expression can be assessed in these sections by immunohistochemistry using a CR-1-specific antibody we have prepared against a synthetic peptide (first described in [10]).

As reported in the first year annual report, there were technical difficulties with the retroviral system chosen for the gene transduction experiments proposed throughout this grant application. Transduction of mammary epithelium was possible, but expression of the transgene was restricted to the duct end buds. It was not possible to make a completely transgenic epithelial tree. Thus, though SOW Tasks 1-3 were attempted with the retroviral vectors, no conclusive results were obtained.

The issue at hand is how to alter CR-1 expression or levels in the developing mammary gland. Since the retroviral strategy does not appear viable, we are left with choosing another vector (e.g. adeno-associated virus) or attempting to add CR-1 protein in some sort of slow-release system (e.g. Elvax pellet). There is one report [18] that implantation of a peptide consisting of only the EGF domain of CR-1 in Elvax pellets affected the growth and morphology of the developing epithelial tree. However, full-length native CR-1 protein has still not been isolated, and that may give different results than those observed with only a portion of the protein. Therefore, we have chosen to attempt to purify native CR-1 and add it back to the developing mammary gland. We have had some success using immunoaffinity columns (Figure 1), though purifying enough protein to use in vivo presents a problem. We used Hi Trap-NHS activated columns (Pharmacia Biotech, Uppsala, Sweden) with purified CR67 antibody used throughout this study. Cells (MCF-7 cells in this case, as they make a large amount of CR-1) were lysed in RIPA buffer and the lysate was clarified by centrifugation and

passage through a 0.45 μ m filter. It was verified by western blot that these treatments did not affect the level of CR-1 in the lysate (not shown).

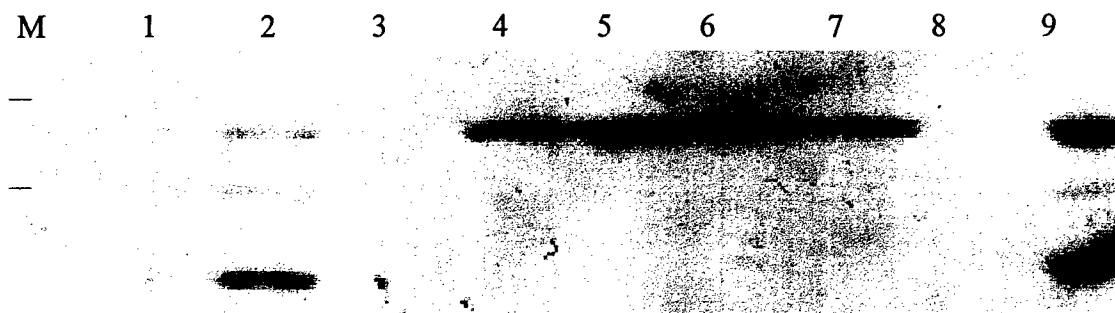


Figure 1 - Isolation of CR-1 via CR-67 Immunoaffinity Column .

Western Blot using CR-67 as the primary antibody. Lane M, markers 57, 43, 29 kD; Lane 1, wash prior to loading the column; Lane 2, flowthrough fraction after loading with cell lysate; Lane 3, wash after loading; Lanes 4-8, serial fractions from the column after loading 100 mM glycine (pH 2.5); Lane 9, cell lysate prior to loading on the column.

As seen in Figure 1, only one of the three CR-67-reactive species is purified by the column, perhaps due to conformational differences and relative availability of the CR-67 epitope. We are currently attempting to develop better expression systems to isolate more CR-1 protein to use in the developing mammary gland. This will be useful not only for this work, but in any future functional studies where intact glycosylated protein is desired.

One key open question regarding CR-1 is what proteins it interacts with on target cells. No receptor has been identified to date, though it has been established that CR-1 does not bind to the ErbB receptors [19, 20]. We were able to purify enough CR-1 to perform a far-western blot on mouse fibroblasts, mouse mammary epithelial cells, and human breast cancer cells. (Figure 2). Briefly, lysates from the target cells were subjected to SDS-PAGE and transferred to PVDF. Duplicate blots were exposed to either purified CR-1 or BSA, washed, then probed with anti-CR-1 antibody (CR-67) in a standard western blot. Bands that are present only on the blots where CR-1 peptide was used prior to the western blot indicate the presence of CR-1 binding proteins.

This isolation of CR-1 also resulted in some loss of the CR67-antibody from the column, thus the two bands observed in the fraction only lanes. The essential observations are the presence of a new CR-1-binding band at >200 kD in the murine mammary epithelial cells and MDA-MB-231 cells (denoted by arrows). In addition, a band at ~ 80 kD is found in the mouse mammary cells (arrows). Though the MCF-7 cells make a large amount of CR-1 protein, there are no differences in the bands present in the far-western and control blots. It is possible that one of these bands represents a CR-1 receptor. It has been recently reported that some crosslinking experiments with

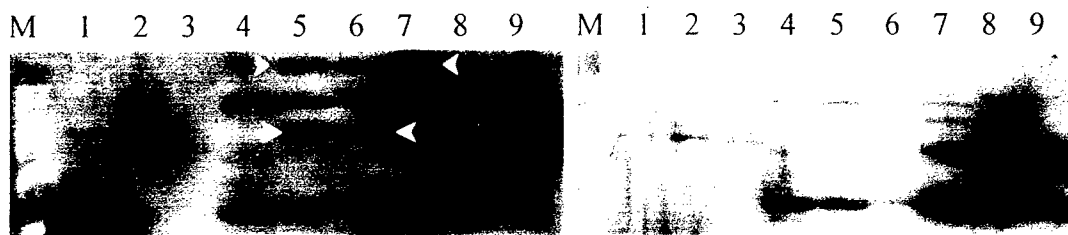


Figure 2 - Far Western using CR-1 immunopurified fraction 4 (left) and control (right) Far Western Blot using immunopurified CR-1 (left) or BSA (right), followed by CR-67, antibody. Lane M, (white bands) 184, 145, 99, 70, 57, 43 kD ; 2, Fraction 3; 3, Fraction 4; 4, Fraction 5; 4, mouse fibroblasts (GPE86, 3T3 derivative); 5, CL-S1; 6, +SA; 7, MDA-MB-231; 8&9, MCF-7.

non-glycosylated, partially truncated CR-1 showed CR-1 binding to 130 kD and 60 kD proteins [21]. Perhaps the differences observed by us are due to the use of fully glycosylated CR-1 or the use of a far-western rather than chemical cross-linking.

T.O. 1b. For precise quantification of CR-1 protein levels, the experiment will be repeated, and glands will be harvested for protein analysis by western blot. CR-1 will be detected using the same antibody as for immunohistochemistry.

This will not be performed unless we are able to isolate enough CR-1 to use in slow-release pellets (SOW Task 4).

T.O. 2 We have designed and tested a hammerhead ribozyme [22, 23] that recognizes nucleotides 12-28 of the murine CR-1 mRNA and cuts after the GUC triplet at nucleotides 18-20. A search of the GenBank database (FASTA, GCG Wisconsin Package) revealed no significant nucleotide sequence identity to any other published sequence, including related EGF family members.

As described in the first year annual report (and published [24]), this ribozyme did not act through a catalytic mechanism. It is unclear why the ribozyme is not catalytic. Perhaps it is due to poor folding due to the long regions 5' and 3' of the ribozyme domain in the ribozyme transcript, or it may be due to the site chosen for ribozyme binding. During the course of investigation of the mechanism of ribozyme activity, we were able to obtain a vector for the expression of ribozymes which contains self-cleaving ribozymes which flank the ribozyme of interest 5' and 3' (kindly provided by J. Norris, University of South Carolina Medical School). We have made two additional ribozymes which use this self-splicing system. The first targets the 5' untranslated region at residue 185, and the second targets the coding region at residue 519. We have tested the self-splicing ability of these two constructs and found it to be intact (Figure 3). The expected transcript before cleavage by the self-splicing ribozymes is 249 nt. Cleavage at both sites should generate fragments of 105, 62, and 82 nt. Cleavage at only one of the sites would generate fragment pairs of 167 and 82 nt or 105 and 144 nt. All of these species can be seen in Figure 3. The most prominent bands are at 249, 167, 105, and 82. A light band may be seen at 144, and the band at 62 is either not present or too faint to see. This may

indicate some problem in one of the cleavages, but the ribozyme should be active whether or not the cleavage is taking place.

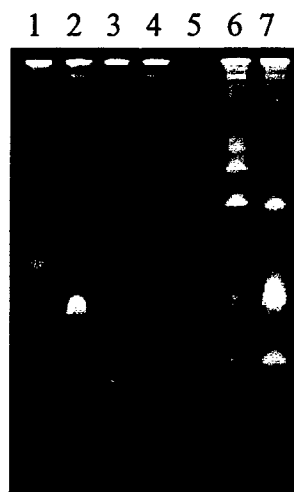


Figure 3 - Self-cleavage of ribozyme constructs.
Lanes 1-4, markers: 134, 104, 77, 56 nt; Lane 5, skip; Lane 6 ribozyme 185; Lane 7, ribozyme 519.

We have also tested these ribozymes in an in vitro cleavage assay. The expected bands for the uncleaved CR-1 transcripts are 784 nt for pECR-1 and 569 nt for pECR-1-5'UTR. The pECR-1 construct contains the full-length cDNA for the murine CR-1 clone from the transcriptional start through the coding region. The pECR-1-5'UTR construct is missing a large portion of the 5' untranslated region (including the recognition site for ribozyme 185). The expected fragment sizes for CR-1 are shown below.

Template	Ribozyme	Fragment Sizes
pECR-1	none	784
pECR-1-5'UTR	none	569
pECR-1	185	250, 534
pECR-1-5'UTR	185	569
pECR-1	519	584, 200
pECR-1-5'UTR	519	369, 200

We found that the 519 ribozyme cleaves well in vitro, while the 185 ribozyme cleaves poorly, if at all (Figure 4). Because the complete ribozyme transcripts were added to the reactions, the smaller ribozyme pieces may also be present. There were many non-specific transcripts present in the samples, but it is apparent that the 519 ribozyme effectively cut both the long and shortened CR-1 transcripts, generating the expected new fragments (indicated by the arrows on the figure). The 185 ribozyme did not apparently cleave the transcript.



Figure 4 - Ribozyme in vitro cleavage reactions.

Lane 1, pECR-1; Lane 2, pECR-1-5'UTR; Lane 3, pECR-1 + Ribozyme 185; Lane 4, pECR-1-5'UTR + ribozyme 185; Lane 5, pECR-1 + Ribozyme 519; Lane 6, pECR-1-5'UTR + Ribozyme 519.

The CL-S1 cell line was derived from a preneoplastic BALB/c mammary nodule, and the -SA, and +SA target cell lines were isolated from a spontaneous adenocarcinoma derived from the same nodule ([25]). CL-S1 cells are anchorage-dependent and do not form tumors in syngeneic hosts; -SA cells do not form colonies efficiently in soft-agar, but will form tumors in syngeneic hosts after injection *in vivo*; +SA cells display efficient colony formation *in vitro*, and are neoplastic and metastatic (pulmonary metastases after intravenous injection) when inoculated into syngeneic hosts *in vivo* [25]. CR-1 expression is strong in +SA cells, slightly lower in -SA cells, and low in CL-S1 cells (unpublished results). A CR-1 over-expression retroviral vector has also been constructed (CA1CR) and used to infect CL-S1.

T.O. 2a. Changes in the growth phenotype of the transfected clones which show the greatest difference in CR-1 levels from the parental line will be evaluated *in vitro* by examining growth rate and the relative efficiency of colony formation in soft agar [25] in triplicate cultures of the various transfected lines. Differences in amount of cellular DNA and colony numbers in soft agar will be analyzed using ANOVA and Fisher's PLSD.

As stated in last year's report, overexpression of CR-1 in CL-S1 cells did not cause any significant change in growth or ability to form colonies in vitro. Transfection of -SA cells with the original ribozyme was effective at reducing CR-1 expression, but neither overexpression nor underexpression affected growth on plastic or in soft agar. Transfection of +SA cells did affect growth in soft agar as shown in Figure 5. Infection with the retroviral vectors appeared to decrease colony formation from the control, though to a lesser degree for the CR-1-containing vector. Transfections with 10 micrograms of plasmid had the most dramatic effects. Transfection with the overexpression vector dramatically increased colony formation, while transfection

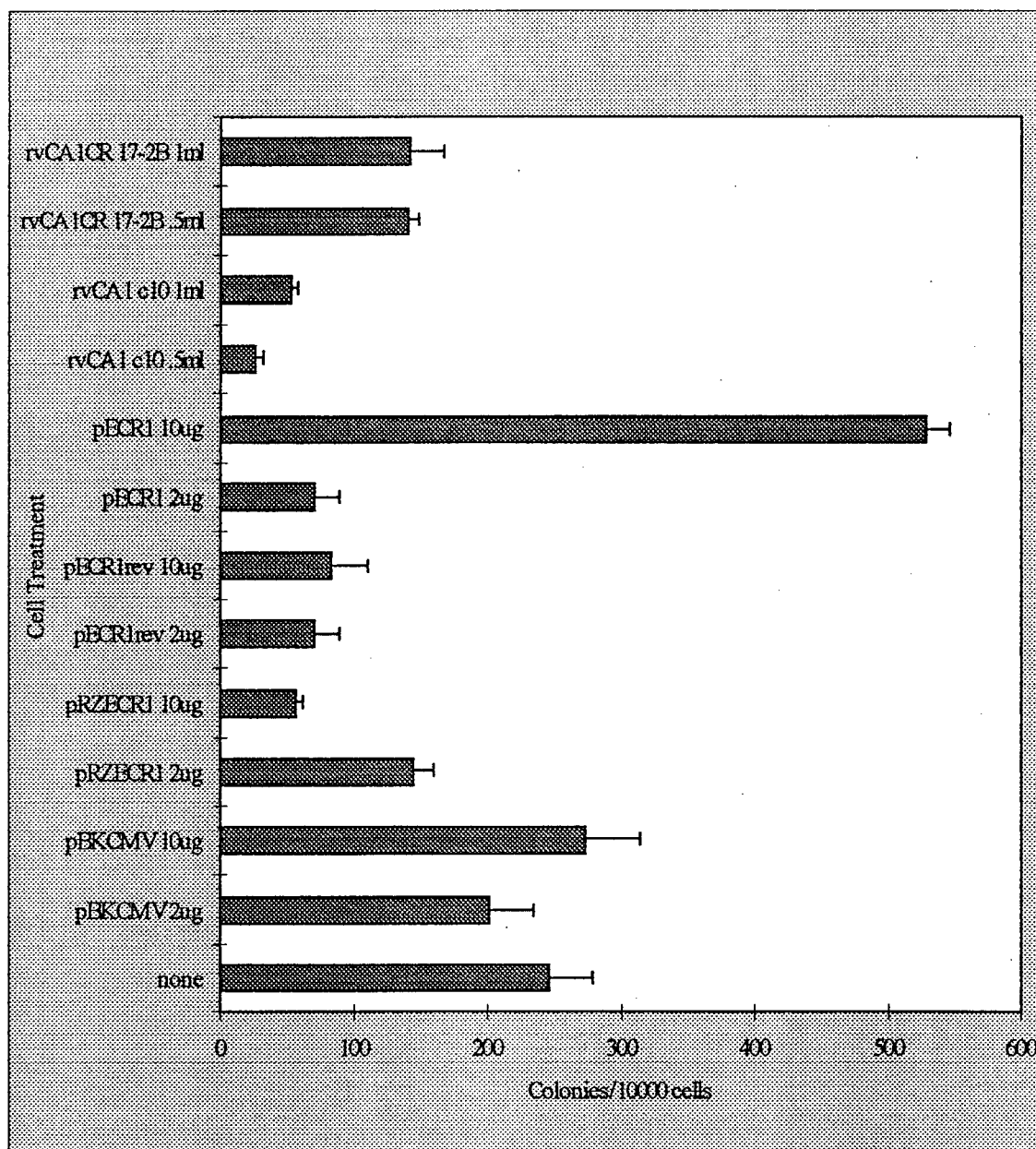


Figure 5 - Formation of colonies in soft agar.

All treatments are to +SA cells. Plasmid transfections were with either 2 or 10 micrograms of plasmid, and retroviral infections were with either 0.5 or 1 ml of filtered producer cell supernatant. Reagents are as follows: rvCA1CR 17-2B: retrovirus carrying CR-1; rvCA1c10, retrovirus without CR-1; pECR1, eukaryotic expression vector carrying CR-1; pECR1rev, eukaryotic expression vector carrying CR-1 in the antisense orientation; pRZECR-1, eukaryotic expression vector carrying a CR-1-specific ribozyme; pBKCMV, eukaryotic expression vector with no insert.

with either the ribozyme (best) or antisense (good) vectors reduced colony formation. Transfection with the expression plasmid alone had no significant effect on colony formation. These experiments essentially conclude Tasks 5-7, though we will repeat them with the new vectors under development (discussed later).

T.O. 2b. The clones analyzed in T.O. 2a will be implanted into syngeneic mice for *in vivo* analysis. Five $\times 10^5$ cells will be injected into epithelium-free (cleared) mammary fat pads of 6-8 week old female BALB/c mice. Six to eight week old animals will be used because (1) the fat pads are larger in these animals, so the surgical implantations are mechanically easier to perform; and (2) at three weeks of age, the mice are still in puberty, and we wish to minimize possible effects of the hormonal state of the animal on the results. The mice will be palpated twice per week for tumor development and tumor size will be measured with a caliper. In untreated mice, +SA-derived tumors are typically palpable after 14-21 days [25]. Therefore, mice will be sacrificed at the end of 4 weeks, unless they are moribund prior to that time, in which case they will be sacrificed immediately and the date of sacrifice noted. Mammary tumors will be further evaluated after fixation, clearing and staining of whole mammary glands. Both whole mounts and sections from them will be analyzed with respect to outgrowth pattern of the transplanted cells (normal vs. hyperplastic). Differences in tumor size (and number if separate tumors are generated) will be compared among all cell types by ANOVA and Fisher's PLSD.

The same cell populations (1×10^5 cells) will be injected into the tail vein of 6-8 week old BALB/c mice to evaluate the potential for the formation of pulmonary metastases [25]. Three mice will be used for each of the cell populations, and they will be sacrificed at 4 weeks after inoculation. Differences in pulmonary tumor number and size will be analyzed using ANOVA and Fisher's PLSD.

Last year we reported on the transfer of -SA/pRZECR-1 clones into mice. It is still not clear if the tumors observed were due to reversion of these cells or recruitment of other cells into the tumors (all of which were CR-1-positive). These experiments will be repeated with the new ribozymes as soon as the constructs are finished.

T.O. 3a. Five $\times 10^5$ +SA cells will be implanted into the cleared #4 fat pads of sixteen 6-8 week old mice. The position of the +SA cells in the gland will be marked on the skin surface to facilitate later injection of viral suspensions. The glands will be infected with 2×10^6 cfu (in 2 injections of 25 μ l at 4×10^4 cfu/ μ l) of the β -gal-expressing CA1 retroviral vector in the presence of 80 μ g/ml polybrene [27]. Injections will begin from 0-14 days after the implantation of the tumor cells and will be repeated every other day until day 18 all mice will be sacrificed on day 28 after implantation of tumor cells and the glands will be removed for analysis. Glands will be fixed and stained for β -gal. Tumor size, percentage of infected (β -gal positive) tumor cells, percentage of infected surrounding cells, and distance of viral spread from the injection point will be recorded. The optimal time after implantation of the tumor cells and the maximum time after implantation (and therefore maximum tumor size) allowing efficient infection with the vector will be determined. The planned viral inoculum will result in a vector: target ratio of 4:1 at the time of tumor cell implantation, if all of the transplanted tumor cells survive. Should these vector:target

ratios prove insufficient for efficient infection of the tumor, we can either reduce the initial tumor cell dose or attempt to further increase the viral titer.

SOW Tasks 9 and 10; these experiments have not yet begun and due to problems with the retroviral constructs will not be attempted as written.

T.O. 4a. The retroviral constructs CA1 and CA1CRZ used in T.O. 1 will be used to treat +SA tumors *in vivo*. +SA tumors will be generated in the cleared #4 mammary fat pads of fifteen 6-8 week old mice as described in T.O. 3. Ten of the mice will be infected with CA1 and CA1CRZ at the optimal conditions determined under T.O. 3 (CA1 on one side CA1CRZ on the other). The five remaining mice will receive only DME containing 80 µg/ml polybrene (in both sides) as a control (see Table 4 for a summary). This design will allow a paired comparison (controlling for host variation) between CA1 and CA1CRZ, and will allow a general comparison (ANOVA + Fisher's PLSD) among all three groups. Glands will be scored for tumor size, percentage of infected tumor cells, and percentage of infected surrounding cells (determined by β-gal staining). A group size of ten (glands) for each treatment is used here rather than the six used in T.O. 2, because reliance on infection of the tumor cells rather than using a stably transfected tumor cell line may increase experimental variability.

SOW Task 11; these experiments have not yet begun but will be attempted with the new vectors as they are developed.

New vectors: We have cloned the murine and human CR-1 genes as well as the new 185 and 519 self-splicing ribozymes into the pAdTrackCMV shuttle vector for use with the AdEasy system developed in Vogelstein's laboratory [26]. These shuttle vector constructs have been sequenced and we are proceeding with the construction of the recombinant viruses. We have verified that expression of transgenes from adenoviral vectors continues for at least 35 days after infection of +SA cells, and that at an moi of 50, it is possible to infect essentially 100% of cells. We plan to use these vectors for Tasks 9-11 after verifying their effectiveness at reducing or increasing CR-1 expression. One advantage of this approach is that it is unnecessary to clone cells after infection, as essentially all will be expressing the transgene or ribozyme. A disadvantage is that this is a non-replicating vector and will be lost over time due to cell division. This means that this vector is unacceptable for the experiments described in T.O. 1, but should work for the shorter term experiments in T.O. 3 and T.O. 4.

Key Accomplishments for Years 1 and 2

1. It is possible to eliminate CR-1 protein expression through the use of a ribozyme-like molecule, even without catalytic activity. This decrease is substantially better than that previously observed with antisense strategies [13]. This may indicate that RNA molecules with strong secondary structure can be targeted to the extreme 5' end of a message to obtain excellent reduction or elimination of expression.

2. Overexpression of Cripto-1 in the preneoplastic line CL-S1 did not cause an increase in growth, colony formation in soft agar, or tumor formation *in vivo*.
3. Elimination of CR-1 expression in -SA cells did not affect growth rate or colony formation *in vitro*. The *in vivo* results are not yet clear.
4. It is possible to transduce nascent mammary epithelium with injection of either retrovirus or retrovirus-producing cells. However, expression appears to be restricted to actively dividing cells, not the entire epithelial tree.
5. It is possible to significantly alter the ability of +SA cells to form colonies in soft agar by transfecting them with CR-1 overexpression and underexpression vectors. The ribozyme vector appeared to work slightly better than the antisense vector. The retroviral vector inhibited colony formation with or without CR-1 expression.
6. It is possible to purify CR-1 using an antibody affinity column, though the amount of protein purified must be increased to be useful as a reagent.
7. Use of the purified CR-1 in a far-western blot showed that there are at least two CR-1 binding proteins present in human and mouse mammary epithelial cells.
8. We have developed at least one more CR-1-specific ribozyme. This one will cleave CR-1 transcripts *in vitro*.

Reportable Outcomes

1. Kintner, R.L. and Hosick, H.L. 1998. Reduction of Cripto-1 expression by a hammerhead-shaped RNA molecule results from inhibition of translation rather than mRNA cleavage. *Biochem. Biophys. Res. Comm.* **245**:774-779.

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Reduction of Cripto-1 Expression by a Hammerhead-Shaped RNA Molecule Results from Inhibition of Translation Rather Than mRNA Cleavage

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Cripto-1 (CR-1) is a transforming growth factor which has been associated with breast, colon, and pancreatic cancer. Overexpression of CR-1 in non-tumorigenic mouse mammary epithelial cells and fibroblasts results in an increase in anchorage-dependent and -independent growth *in vitro*. Reduction of CR-1 expression in human colon carcinoma or embryonal teratoma cells results in a decrease in growth *in vitro*. In an effort to better define the role of CR-1 in breast cancer, we have developed an underexpression vector for CR-1 to reduce CR-1 levels in a tumorigenic mouse mammary epithelial cell line (-SA). This vector specifically targets the expression of the murine homolog of CR-1 in murine cancer lines and utilizes a hammerhead ribozyme-like structure directed toward the extreme 5' end of the Cripto-1 mRNA. We dramatically reduced expression of CR-1 through the expression of this RNA. This is the first use of a ribozyme-like molecule to alter Cripto-1 expression. This ribozyme-shaped molecule appears to act principally through a block in translation. A possible mechanism for this block is described, and its implications for modifying expression of other bioactive proteins are discussed.

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Cripto-1 (CR-1) is a member of the Epidermal Growth Factor (EGF) family. It was originally cloned from human embryonal carcinoma cell line NT2D1 (1), and the murine homolog was later cloned from mouse embryos (2). CR-1 is expressed in 60-80% of colorectal cancers, but in only 0-7% of normal colon mucosal cells (3,4). CR-1 can be detected in several human breast cancer cell lines (3,4), and has been detected in 75-82% of human breast tumors, with none of the protein

identified in adjacent, non-cancerous tissue (3-5). CR-1 has also been found in gastric carcinomas (6) and pancreatic cancer (7).

In addition to the descriptive studies cited above, CR-1 expression has been experimentally altered in certain cell lines, and the results demonstrate that it acts as a transforming growth factor. Overexpression of human CR-1 in immortalized, non-tumorigenic mouse fibroblasts, NIH3T3, resulted in a pronounced increase in anchorage-independent growth *in vitro* (1). Overexpression of the human CR-1 gene in the immortalized, non-tumorigenic mouse mammary epithelial line, NOG-8, resulted in increased anchorage-dependent and -independent growth *in vitro*, but not in tumorigenicity *in vivo* (8). Finally, incubation of two human mammary carcinoma lines and one nontransformed mammary epithelial cell line with synthetic peptides derived from the CR-1 sequence or with conditioned medium from CHO cells transfected with the human CR-1 gene resulted in increased anchorage-dependent growth *in vitro* (9).

In addition to these overexpression studies, responses to reduction of CR-1 expression through the use of antisense RNA oligonucleotides or antisense expression vectors have also been assessed. In human colon carcinoma cells (GEO), reduction of CR-1 expression inhibited anchorage-dependent and -independent growth *in vitro* and reduced tumorigenicity *in vivo* (10). Also, reduction of CR-1 expression in NT2D1 embryonal carcinoma cells resulted in decreased anchorage-dependent and -independent growth *in vitro* (11).

Although these expression reduction studies paint a clear picture of the importance of CR-1 expression in colon cancer cells and embryonic teratoma cells, the data for breast cancer cells are less clear. To gain further insight into the potential role for CR-1 in mammary tumorigenesis, we have chosen to examine the effect of reducing CR-1 expression in a tumorigenic mouse mammary epithelial cell line, -SA. This cell line,

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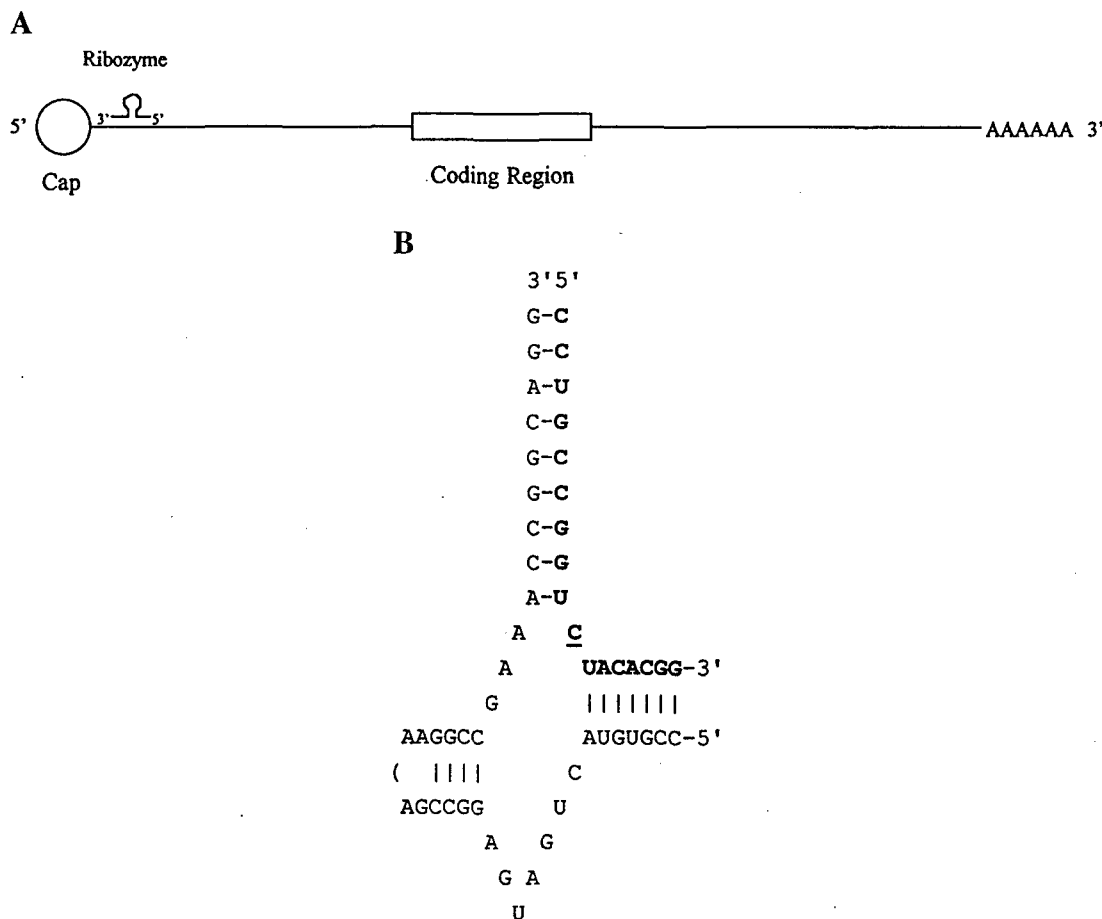


FIG. 1. CR-1-specific hammerhead ribozyme. (A) Diagram of the processed CR-1 mRNA, showing the ribozyme recognition sequence with respect to the 5' cap, coding region, and polyA tail. (B) Sequence of the ribozyme and its presumed binding conformation to the CR-1 message.

which was isolated from a spontaneous mouse mammary adenocarcinoma, does not form colonies in soft agar, but does form tumors *in vivo* (12).

Finally, these experiments focus on providing an effective way to decrease murine CR-1 expression in mouse cells. Though the human and murine genes exhibit 80% homology overall (2), it will be necessary to carefully examine the effects of altering CR-1 expression using protein and cells from the same species, rather than simply using the human CR-1 protein in all cells, as has been done to date.

MATERIALS AND METHODS

Plasmids and constructs. The Cripto-1-specific hammerhead molecule was synthesized as two complementary single-stranded DNAs, 43 nucleotides long (Eppendorf Custom Oligonucleotides, Madison, WI). Two μ g of each oligo were mixed and heated to 75°C for 10 minutes. They were allowed to cool slowly to 4°C and then electrophoresed on a 3% Metaphor (FMC, Rockland, ME) agarose gel to verify annealing. The annealed molecule was then ready for cloning into an expression vector.

The basic expression construct used in this study was pBKCMV (Stratagene, La Jolla, CA). This is a eukaryotic expression vector

which expresses the *neo* gene from an SV40 promoter to allow for selection of stable transfectants, and the cloned sequence from the cytomegalovirus (CMV) immediate early promoter to provide high levels of expression. The hammerhead construct, pRZECR-1, was made by cloning the Cripto-1-specific ribozyme sequence between the *Bam*HI and *Eco*RI sites in pBKCMV. This puts the hammerhead sequence under the control of the strong CMV promoter. Correct insertion was established by restriction enzyme analysis and verified by sequencing (Applied Biosystems automated sequencer, Laboratory for Biotechnology and Bioanalysis, WSU, Pullman, WA).

Cells and cell culture. The -SA cell line was isolated from a spontaneous adenocarcinoma that developed within a preneoplastic mouse mammary nodule (12). This line does not form colonies in soft agar, does form tumors in syngeneic hosts after injection *in vivo*, and is not metastatic. Unless otherwise indicated, all cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Sigma, St. Louis, MO) supplemented with 10% bovine calf serum (Hyclone, Ogden, UT). Cells were maintained at 37°C in a 5% CO₂ environment.

Transfection of pBKCMV (control) and pRZECR-1 into mammary tumor cells was accomplished using Lipofectamine (Gibco/BRL, Gaithersburg, MD), according to the manufacturer's instructions. Two to ten μ g of plasmid was used with 10 μ l of Lipofectamine for each 35 mm dish of cells. Transfections were performed in the absence of serum (Optimem medium, Gibco/BRL). Transfected cells were refed with DMEM supplemented with 10% bovine calf serum after 24 hr. Stable transfectants were selected, beginning 72 hr after the transfection, by growth in the presence of 800 μ g/ml Geneticin

(G418, Gibco/BRL). At least 12 G418-resistant clones were isolated from each transfection of -SA cells for evaluation of mRNA and protein production.

Production of the CR-1 antiserum (CR-67). A polyclonal anti-CR-1 antiserum was made by immunizing rabbits with a synthetic peptide (CPPSFYGRNCEHDVRKE) which has been demonstrated to elicit effective CR-1 specific antibodies (4); this antibody is later called anti-CR-67 (9). This peptide is 100% conserved between human and murine CR-1 (2). The polyclonal antiserum was produced by Quality Controlled Biochemicals, Inc., Hopkinton, MA. The peptide was synthesized, purified, conjugated to KLH, and injected into rabbits. Animals were boosted and bled, and the activity of the serum was verified by ELISA against the peptide.

Western blotting. Cells were grown to 90% confluence in 10 cm tissue culture dishes, rinsed with PBS (137 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4 , 2 mM KH_2PO_4), then lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris, pH 7.5) at 4°C. Samples were sonicated on ice and frozen at -20°C. Identical cell equivalents (roughly 300,000 cells) of lysates were diluted 1:1 with 2× Laemmli loading buffer (100 mM Tris, pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol), supplemented with β -mercaptoethanol to a final concentration of 5%, heated to 98°C for 10 minutes, and electrophoresed through replicate discontinuous SDS-polyacrylamide gels (5% stacking, pH 6.8, 12% resolving, pH 8.8). After electrophoresis, one gel was rinsed in distilled H_2O , and electroblotted to a PVDF membrane (MSI, Westboro, MA), while the other was stained with Coomassie Brilliant Blue to verify identical lane loading. After the transfer, the blot was blocked for 1-2 hours in TBS-Tween (TBST, 100 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween-20) with 5% nonfat dry milk. The blot was incubated for 1 hour with a 1:1000 dilution (in blocking solution) of the CR-1 polyclonal antiserum (CR-67), washed 3 × 10 minutes in TBST, incubated for 1 hour in a 1:3000 dilution of goat-anti-rabbit-alkaline phosphatase antibody (Bio-Rad, Hercules, CA), washed 3 × 10 minutes in TBST, and incubated for 5 minutes with the CDP-Star chemiluminescent substrate (New England Biolabs, Beverly, MA). Bands were detected by exposure of x-ray film.

RT-PCR. Total RNA was isolated from the -SA/pBKCMV control clone #14, -SA/pRZECR-1 clones #4, 5, 11, 18, 23, and 24, -SA cells, and +SA cells using Ultraspec RNA Isolation Reagent (Biotecx, Houston, TX). mRNA (polyA⁺) was isolated from the total RNA preps using oligo dT/magnetic bead separation (PolyATtract, Promega, Madison, WI). Approximately 1 ng of polyA⁺ mRNA was reverse transcribed with MoMuLV RT (Promega, Madison, WI), and 1/10 of each RT reaction was amplified using *Taq* polymerase (Promega, Madison, WI) and the following primers (fwd, CCCATCCCTGCGGTCT; rev, AAGCGAGGCGCCAGCTAG); this generated the expected fragment of 716 bp. Control reactions (1/10 of each RT reaction) using actin primers generated the expected 360 bp fragment. RT-PCR reactions were electrophoresed through 1.5% agarose-TAE gels, stained with ethidium bromide and photographed. The photographs were scanned and then analyzed for band intensities (Intelligent Quantifier, BioImage, Ann Arbor, MI).

RESULTS

Construction of the CR-1-Specific Ribozyme

Using the published sequence of mouse CR-1 (2), we designed a hammerhead ribozyme (13,14) which recognizes nucleotides 12-28 of the murine CR-1 mRNA and should cut after the GUC triplet at nucleotides 18-20. This site was chosen for three reasons. First, an RNA folding analysis (mfold, by Zuker and Jaeger, using the GCG Wisconsin Software package) of the first 1050

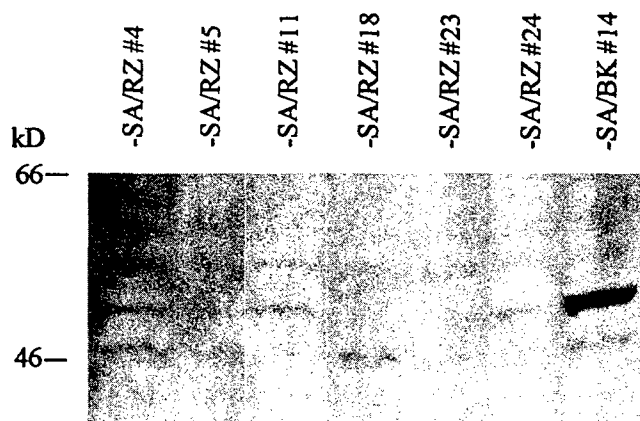


FIG. 2. Western blot of -SA clones transfected with pBKCMV or pRZECR-1. Lane kD, molecular weight in kilodaltons; 2-6, -SA clones transfected with pRZECR-1; 7, -SA control clone transfected with pBKCMV.

bases of the mRNA showed that this site should be accessible. Specifically, residues 7-17 and 22-25 form single-stranded loops in the 14 best structure predictions. Second, this site is at the extreme 5' end of the mRNA, and cleavage or binding at this site will thus result in the immediate elimination of CR-1 translation. Cleavage further along the message might allow production of a truncated, but still bioactive, peptide prior to degradation of the message. Finally, it has been shown that stem-loops engineered into an RNA sequence within 12 nt of the 5' cap can prevent the 40S ribosomal subunit from binding to the mRNA (15). Binding of the ribozyme at this site may provide secondary structure that will inhibit binding of the 40S subunit, even if the mRNA is not cleaved. The 39-nucleotide hammerhead molecule was synthesized as two complementary 43-nucleotide strands with 5' overhangs. These overhangs allowed for efficient directional cloning into the pBKCMV eukaryotic expression vector. Proper insertion was determined by restriction analysis (data not shown) and verified by sequencing. A diagram of the CR-1 message and ribozyme is shown in Figure 1A, and the folded hammerhead, bound to the CR-1 substrate mRNA is shown in Figure 1B.

Testing of the Ribozyme

The control expression vector (pBKCMV) and the ribozyme expression vector (pRZECR-1) were transfected into -SA cells using Lipofectamine. Bulk cultures were selected for the presence of the vectors with G418 (Geneticin), and at least 12 stably-transfected clones were picked from each transfection. For each transfection, protein was harvested from twelve clones and CR-1 expression was tested by western blot using the CR-67 antiserum. Figure 2 shows one -SA/BKCMV control clone and 6 -SA/RZECR-1 clones. -SA/RZECR-1 clones # 5, 18, and

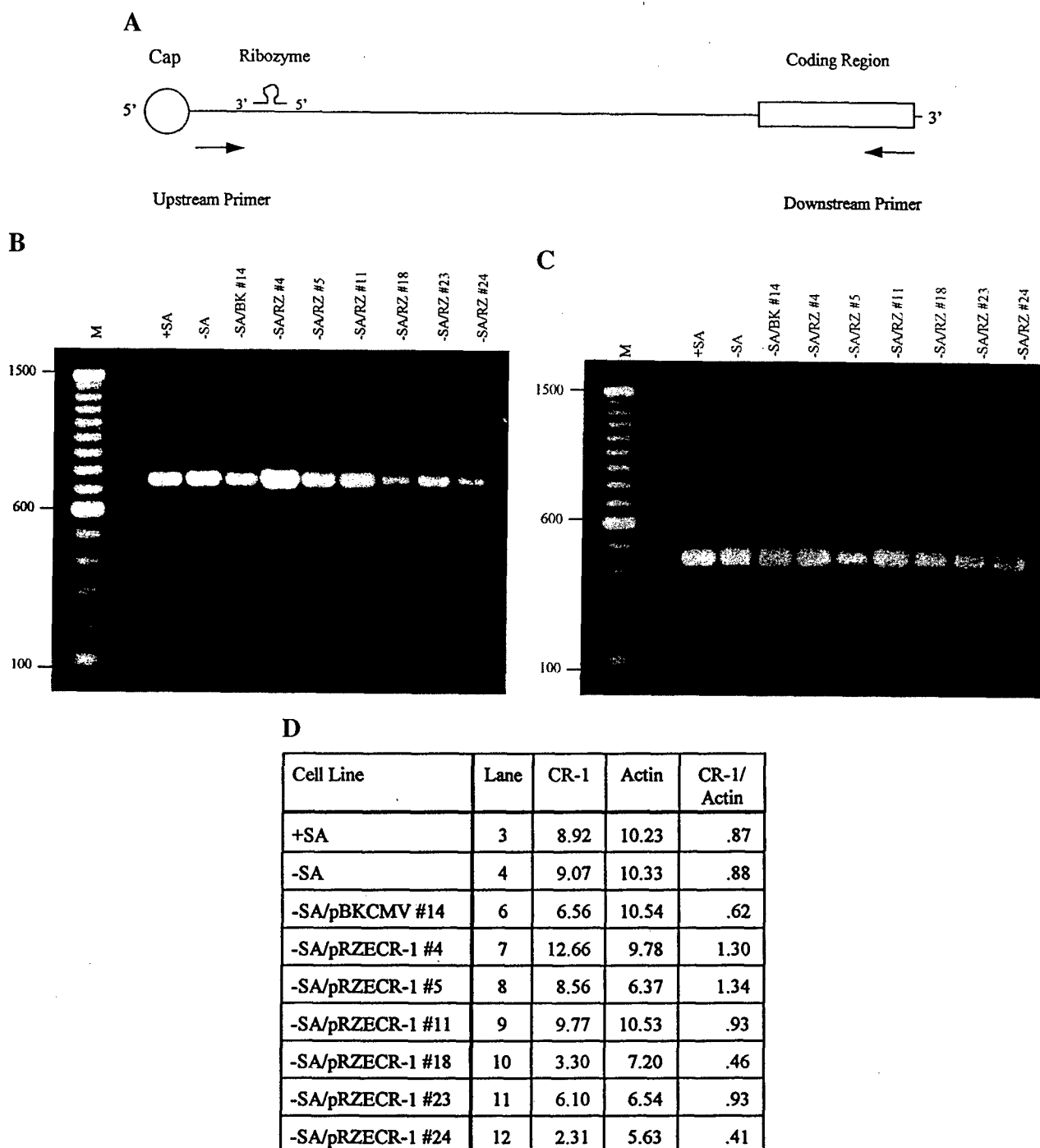


FIG. 3. RT-PCR Analysis of mRNA expression in transfected -SA clones. (A) Diagram of the 5' portion of the CR-1 mRNA, showing the 5' cap, ribozyme binding site, coding region, and PCR primers. (B) Electrophoretic analysis of RT-PCR using the CR-1-specific PCR primers. Lane 1, 100 bp marker; 2, blank; 3-4, untransfected controls; 5, -SA control clone transfected with pBKCMV; 6-11, -SA clones transfected with pRZECR-1. (C) Electrophoretic analysis of RT-PCR using actin-specific PCR primers. Lane 1, 100 bp marker; 2, blank; 3-4, untransfected controls; 5, -SA control clone transfected with pBKCMV; 6-11, -SA clones transfected with pRZECR-1. (D) Relative intensities of RT-PCR bands in panels B and C.

23 show complete loss of expression of the CR-1 protein, and -SA/RZECR-1 clones # 4, 11, and 24 show near-complete loss of expression. All -SA/pBKCMV clones showed similar levels of CR-1 expression (data not shown).

Mechanism of Activity of the Ribozyme

It was apparent from the western blot that the ribozyme was effective at eliminating CR-1 expression, but

not whether this was through an enzymatic or other mechanism. The cleavage site for the message was too near the 5' end of the CR-1 message to distinguish cleaved from non-cleaved message on a Northern blot. Therefore we opted for a reverse transcriptase-PCR strategy. Primers were designed that spanned the ribozyme cut site and would only amplify a message that had not been cleaved by the ribozyme (Figure 3A). As shown in Figure 3B, all clones make uncleaved CR-1 message. As shown in Figure 3C, most reactions show similar levels (less than 2-fold difference between lanes) of actin transcript. -SA/RZECR-1 clones #5, #18, #23, and #24 show slightly lower levels of actin, but these variations are smaller than those seen with CR-1 (up to 5.5-fold difference between lanes). After analyzing the bands densitometrically (Figure 3D), and normalizing the CR-1 levels to the actin ones, it appears that there are reduced levels of CR-1 message in clones -SA/pRZECR-1 #18 and 24. Amplification of polyA⁺ RNA samples without reverse transcription did not yield any fragments (data not shown). Given the data in Figures 2 and 3, it appears most likely that this hammerhead ribozyme is reducing the levels of CR-1 protein primarily through a non-cleavage mechanism.

DISCUSSION

While both antisense RNA and ribozymes can effectively reduce expression of target genes (for example (10,11,14,16-19)), we originally chose to develop a ribozyme because of its catalytic nature. Antisense oligonucleotides must generally be produced in stoichiometric amounts to eliminate synthesis of the target protein (20), while a single ribozyme molecule may cleave multiple copies of the target RNA (21,22).

We have now demonstrated that a hammerhead molecule specific for the extreme 5' region of the CR-1 mRNA greatly reduces the expression of CR-1 in a tumorigenic mouse mammary epithelial cell line. This construct has excellent activity. However, the apparent method of action of this molecule requires some consideration. The first question that must be addressed is the apparent lack of cleavage activity. Many studies have shown that the length of the ribozyme and target molecules can have dramatic effects on the efficiency of cleavage (see (23, 24) for an example of each). The expression construct used in this system produces a ribozyme transcript of approximately 800 nt, only 40 of which constitute the ribozyme itself. These extraneous sequences may result in altered folding or binding which inhibit cleavage activity.

The second question to be addressed is how this molecule is so effective at eliminating CR-1 protein expression. Based on an assessment of the 5' untranslated region sequence, CR-1 may be translationally regulated in its native state. It has 2 upstream open reading frames (2), the first of which has a start codon in opti-

mal context (25). In addition, CR-1 appears to act as a transforming growth factor, and many of these types of molecules exhibit translational control of expression (26). The ribozyme, a relatively large (800nt) molecule, binds very near the 5' cap, close enough to inhibit binding of the 40S ribosomal subunit (15). This circumstance may very efficiently block translation, regardless of the ability of the ribozyme to cleave its target.

There is evidence for decreased message levels in some of the ribozyme-transfected clones, and it is possible that this ribozyme acts through mRNA cleavage as well. However, there is no correlation between the apparent degree of cleavage activity and level of protein synthesis suppression. The two clones with the lowest CR-1/actin ratios (18 and 24) come from both the group with nearly complete and the group with complete elimination of CR-1 protein expression. Therefore, it appears that inhibition of translation is the more important mechanism.

In summary, we have shown that a hammerhead ribozyme directed toward the extreme 5' end of the murine CR-1 message is effective at reducing CR-1 protein levels in a tumorigenic murine mammary epithelial cell line. This ribozyme appears to act principally through a non-enzymatic mechanism, though it may also cleave the mRNA in a classic ribozyme fashion. It is highly effective at eliminating CR-1 protein expression, perhaps due to acting through both inhibition of translation and cleavage mechanisms. In general, the targeting of ribozyme-like molecules to the extreme 5' ends of mRNAs may be a very effective method of reducing protein expression in part by reducing the ability of the 40S ribosomal subunit to bind the target message.

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